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Use of the *tac* Promoter and *lacI*^{q} for the Controlled Expression of *Zymomonas mobilis* Fermentative Genes in *Escherichia coli* and *Zymomonas mobilis*†

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The *Zymomonas mobilis* genes encoding alcohol dehydrogenase I (*adhA*), alcohol dehydrogenase II (*adhB*), and pyruvate decarboxylase (*pdc*) were overexpressed in *Escherichia coli* and *Z. mobilis* by using a broad-host-range vector containing the *tac* promoter and the *lacI*^{q} repressor gene. Maximal IPTG (isopropyl-β-D-thiogalactopyranoside) induction of these plasmid-borne genes in *Z. mobilis* resulted in a 35-fold increase in alcohol dehydrogenase I activity, a 16.7-fold increase in alcohol dehydrogenase II activity, and a 6.3-fold increase in pyruvate decarboxylase activity. Small changes in the activities of these enzymes did not affect glycolytic flux in cells which are at maximal metabolic activity, indicating that flux under these conditions is controlled at some other point in metabolism. Expression of *adhA*, *adhB*, or *pdc* at high specific activities (above 8 IU/mg of cell protein) resulted in a decrease in glycolytic flux (negative flux control coefficients), which was most pronounced for pyruvate decarboxylase. Growth rate and flux are imperfectly coupled in this organism. Neither a twofold increase in flux nor a 50% decline from maximal flux caused any immediate change in growth rate. Thus, the rates of biosynthesis and growth in this organism are not limited by energy generation in rich medium.

Zymomonas mobilis represents an excellent model system for metabolic flux control analysis (8, 33). This organism is an obligately fermentative gram-negative bacterium that utilizes the Entner-Douderoff (ED) pathway for glycolysis. More than 95% of the glucose metabolized is converted into ethanol and carbon dioxide with a low ATP yield (29). The glycolytic and ethanologenic enzymes in *Z. mobilis* represent the sole route for energy generation, and together they constitute approximately 50% of soluble cellular protein (2, 3, 34). The biochemistry and kinetics of the enzymes involved have been characterized (6, 21, 30, 31, 34, 38, 41), and some have recently been proposed to form complexes in vivo (1). In contrast to Embden-Meyerhof glycolysis, which exhibits considerable allosteric control, the ED pathway lacks two key allosteric enzymes, namely, phosphofructokinase and an allosteric hexokinase (2, 29, 41). On the basis of biochemical characterizations and the small metabolite pools in *Z. mobilis*, it has been proposed that little allosteric regulation operates within the *Z. mobilis* ED glycolytic pathway (2, 5). Consequently, carbon flux may be limited by the specific activities of pathway enzymes.

Most of the *Z. mobilis* genes encoding ED enzymes and ethanologenic enzymes have been cloned and sequenced (4, 8–12, 24). To facilitate the study of flux control by using these genes, a regulated expression vector is needed for *Z. mobilis* and none have been previously reported. In this article, we describe the modification and use of a broad-host-range vector (18) that allows partial control of plasmid-borne genes. This vector was used to investigate the effects of the ethanologenic genes (*adhA*, *adhB*, and *pdc*) on metabolic activity and growth.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* S17-1(λ*pir*) and DH5α were grown at 37°C in Luria broth (17). *E. coli* KO3 was grown at 30°C in Luria broth containing 5% (wt/vol) glucose. *Z. mobilis* CP4 was grown at 30°C in complex medium as previously described (7), containing nalidixic acid (40 mg/liter) and glucose (100 g/liter). Growth was monitored spectrophotometrically at 550 nm with a Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Aldehyde indicator plates (11) containing 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were used to test for alcohol dehydrogenase (ADH) in recombinant *E. coli*.

DNA manipulations. Standard methods were used for DNA manipulations in *E. coli* (17). Methods for the purification of plasmid and chromosomal DNA from *Z. mobilis* have been described previously (11). Primers were synthesized by using an Autogen 500 oligonucleotide synthesizer (Millipore Corp., Bedford, Mass.). All plasmid constructions were made and characterized in *E. coli* DH5α.

Conjugation of plasmids into *Z. mobilis*. Plasmids were transformed into *E. coli* S17-1(λ*pir*) and conjugated into *Z. mobilis* CP4 by using a modification of the biparental filter mating procedure described by De Lorenzo et al. (14). Samples from overnight cultures of the recipient and donor strains (0.1 ml each) were mixed in 10 ml of 10 mM $MgSO_4$, filtered through a 0.45-μm-pore-size membrane filter under vacuum, and placed on solid mating media containing the following (per liter): glucose, 50 g; yeast extract, 5 g; tryptone, 10 g; K_2HPO_4, 0.2 g; $(NH_4)_2SO_4$, 2.5 g; and agar, 15 g. After incubation (18 h at 30°C), cells were resuspended in 10 ml of 10 mM $MgSO_4$ and spread on selection plates containing the following (per liter): yeast extract, 10 g; KH_2PO_4, 0.3 g; glucose, 20 g; agar, 15 g; tetracycline, 25 mg;

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference[a]
E. coli K-12		
DH5α	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi1 gyrA96 relA1*	BRL
S17-1(λ*pir*)	*thi pro hsdR recA*::RP4 *2-tc*::*Mu-Km*::Tn*7*(λ*pir*)	14
CB1273	*E. coli* HB101(pMMB66EH)	M. Bagdasarian; 18
CB1274	*E. coli* HB101(pMMB66HE)	M. Bagdasarian; 18
E. coli B		
Parent	Prototrophic	ATCC 11303
KO3	*pfl*$^+$ *pfl*::(*pdc*$^+$ *adhB*$^+$ *cat*)	32
Z. mobilis CP4	Prototrophic	33
pUC18	*bla lacI'Z'*[b]	BRL
pLOI135	pUC18 containing *Z. mobilis adhA*; Ap^r	24
pLOI142	pUC18 containing *Not*I-polylinker in *Sma*I site	This study
pBR322	*bla tet*	39
pMMB66EH	RSF1010Δ(*Pst*I-*Pvu*II 2.87 kb) Ω(*lacI*^{q} *tacP rrnB bla Nru*I-*Aha*III 3.0 kb); Ap^r	18
pMMB66HE	Polylinker of pMMB66EH inverted	18
pLOI704EH	pMMB66EH with pUC18 *Eco*RI-*Hin*dIII polylinker	This study
pLOI705EH	pLOI704EH containing *Not*I site	This study
pLOI706EH	pLOI705EH containing a 1.43-kb *Eco*RI-*Ava*I *tet* fragment from pBR322 in the *Pvu*I site within *bla*; Tc^r Ap^s	This study
pLOI707EH	pLOI706EH with pUC18 *Sst*I-*Not*I fragment; Tc^r Ap^r	This study
pLOI704HE	pMMB66HE with pUC18 *Eco*RI-*Hin*dIII polylinker	This study
pLOI705HE	pLOI704HE with a *Not*I polylinker in the *Sma*I site	This study
pLOI706HE	pLOI705HE containing a 1.43-kb *Eco*RI-*Ava*I *tet* fragment from pBR322 in the *Pvu*I site within *bla*; Tc^r Ap^s	This study
pLOI707HE	pLOI706HE with pUC18 *Sst*I-*Not*I fragment; Tc^r Ap^r	This study

[a] BRL, Bethesda Research Laboratories.
[b] Incomplete *lacI* and incomplete *lacZ*.

and nalidixic acid, 40 mg. *Z. mobilis* exhibits natural resistance to nalidixic acid (29). Approximately 30 to 300 recombinants were recovered per plate.

Synthesis of *adhA*, *adhB*, and *pdc* DNA. The *Z. mobilis* genes encoding ADHII (*adhB*) and pyruvate decarboxylase (PDC; *pdc*) were synthesized by using the polymerase chain reaction (35) with *Z. mobilis* chromosomal DNA as a template. Similarly, DNA encoding ADHI (*adhA*) was synthe-

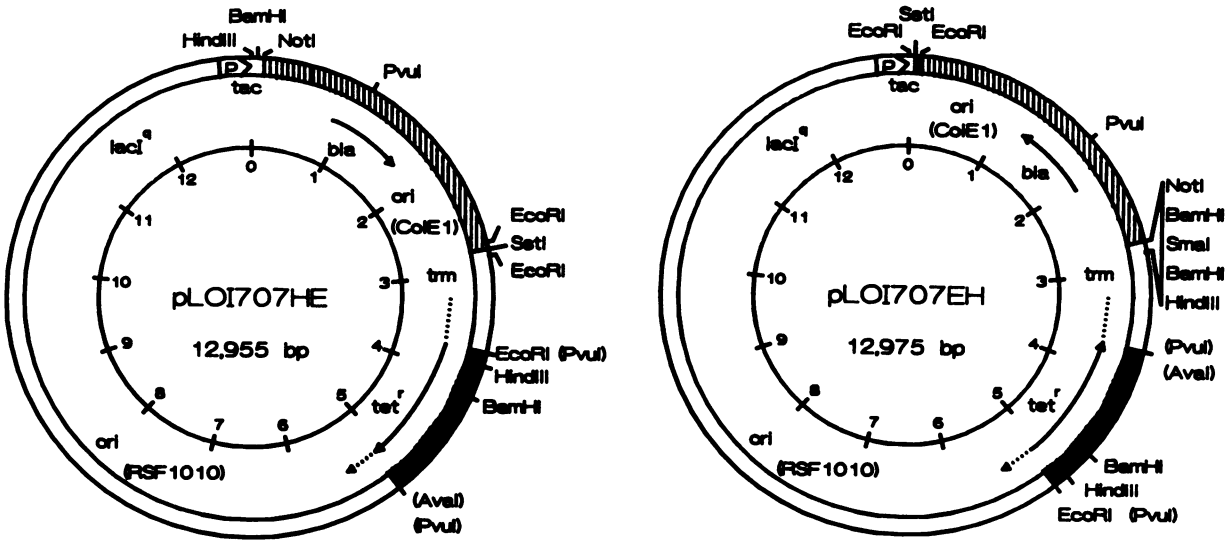

FIG. 1. Physical and genetic maps of pLOI707EH and pLOI707HE. Nucleotide 1 is either the first T residue in the *Eco*RI recognition site (pLOI707EH) or the C residue in the *Hin*dIII recognition site (pLOI707HE). Sequences derived from plasmids pMMB66EH and pMMB66HE are shown unshaded (18, 37). The darkly shaded area represents the 1,427-bp *Eco*RI-*Ava*I fragment from pBR322 containing *tet* which was inserted into the *Pvu*I site of pMMB66EH and pMMB66HE. Insertion of this fragment disrupted the *bla* gene (dashed arrow). The hatched area represents the pUC18 derivative pLOI142 (2,694 bp), inserted into the multiple-cloning site. Restriction sites in parentheses were eliminated as a result of creation of blunt-ended fragments prior to ligation. Abbreviations: *bla*, β-lactamase; trm, transcriptional termination sequence (*rrnB*); Ptac, *tac* promoter; *lacI*^{q}, *lac* repressor gene.

sized by using plasmid pLOI135 as a template (24). The following oligonucleotides were used as primers: 5′ GCG AGC TCC AAC AAA AGG TAG TCA 3′ (5′ end of the *adhA* gene); 5′ GTG GCG GCC GCT AGT GAT GGG TAA AAT 3′ (3′ end of the *adhA* gene); 5′ GCG AGC TCA GTA TGT AGG GTG AGG 3′ (5′ end of the *adhB* gene); 5′ GTG GCG GCC GCT TAG AAA GCG CTC AG 3′ (3′ end of the *adhB* gene); 5′ GCG AGC TCG TGT TTT GAA TAT ATG GAG 3′ (5′ end of the *pdc* gene); 5′ CGG CGG CCG CTT ACG GCT GTT GGC 3′ (3′ end of the *pdc* gene). Primers complementary to the 5′ end of the genes include the native ribosome binding site (underlined) and an *Sst*I site. Primers complementary to the 3′ end of the genes include a *Not*I site. These restriction sites allow the directional insertion of polymerase chain reaction products into the vectors pLOI707EH and pLOI707HE. Neither *Sst*I nor *Not*I sites occur in any of the *Z. mobilis* catabolic enzymes sequenced thus far (4, 8–12, 24).

Enzyme assays. Overnight cultures were diluted into fresh medium and harvested at an optical density at 550 nm (OD_{550}) of about 0.5. To induce expression from the *tac* promoter, this medium contained 2 mM IPTG for *E. coli* S17-1(λ*pir*) recombinants and 5 mM IPTG for *Z. mobilis* CP4 recombinants. Unless stated otherwise, IPTG was included during the growth of inocula for induced cultures.

Cells were permeabilized for enzyme assays as described previously (27). PDC (EC 4.1.1.1) activity was determined as the rate of pyruvate-dependent NADH oxidation at pH 6.5 as described by Neale et al. (31). PDC activity in *E. coli* S17-1(λ*pir*) was corrected for the pyruvate-dependent NADH oxidation activity (catalyzed by lactate dehydrogenase, primarily), as measured in cells containing vector without the *pdc* gene. ADH (EC 1.1.1.1) activity was determined by measuring the ethanol-dependent reduction of NAD^+ at pH 8.5 as described by Neale et al. (30). Two ADH enzymes are present in *Z. mobilis*, ADHI (*adhA*), which contains zinc as a cofactor, and ADHII (*adhB*), which contains ferrous iron as a cofactor. These enzymes represent approximately 20 and 80% of total ADH activity, respectively (27). Total ADH activity was measured by using extracts containing 10 mM ascorbic acid and 0.5 mM ferrous ammonium sulfate. In experiments involving recombinants with CP4(pLOI706EH/*adhA*), ADHI activity was measured by using extracts containing 10 mM 2-mercaptoethanol but lacking ferrous ammonium sulfate and ascorbic acid. ADHII activity was negligible when assayed in these latter extracts. Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured as described by Scopes et al. (38). Specific enzyme activities are expressed as international units (micromoles per minute) per milligram of total cell protein. Protein concentrations were estimated by the method of Lowry et al. (26). For *Z. mobilis* and *E. coli*, an OD_{550} of 1.0 corresponds to approximately 0.23 and 0.21 mg, respectively, of cell protein per ml.

Carbon flux studies. Carbon flux through the glycolytic and ethanologenic pathways was measured as the rate of CO_2 evolution (Q_{CO_2}) during growth. Overnight cultures of *Z. mobilis* CP4 strains were diluted into a 1-liter stoppered flask containing 800 ml of complex medium to provide an initial OD_{550} of 0.1 to 0.2. CO_2 evolution rates were determined volumetrically by collecting the evolved gas in an inverted graduated cylinder, submerged in water acidified with H_2SO_4 to pH 2 (40). *Z. mobilis* cultures were agitated with a magnetic stirring bar at a constant speed (100 rpm) throughout growth and flux measurements. Gas was sampled for periods of less than 5 min, resulting in volumes between 10 and 100 ml. Culture density (OD_{550}) was determined immediately after gas sampling and used to estimate cell protein. Q_{CO_2} is expressed in micromoles of CO_2 per minute per milligram of cell protein (international units per milligram of protein).

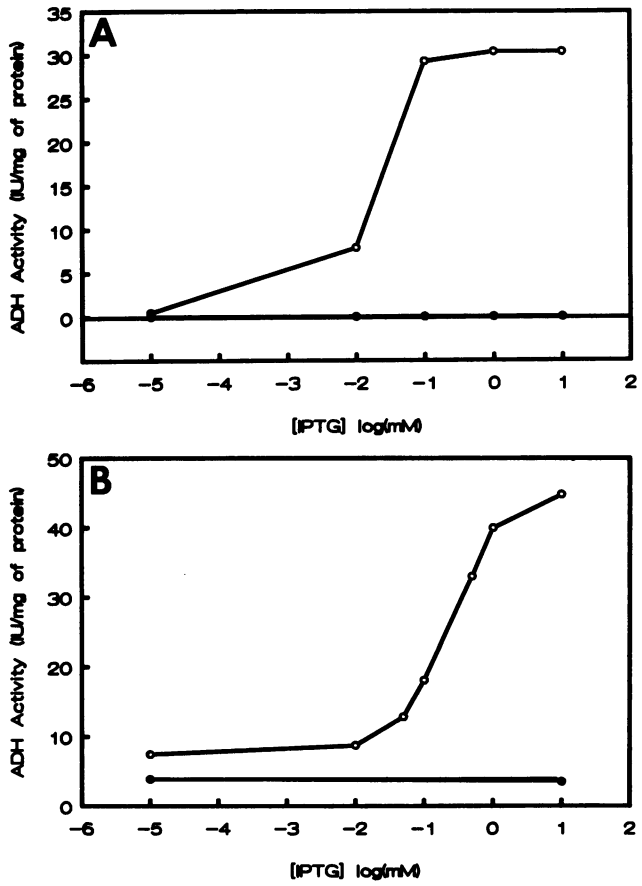

FIG. 2. Effect of growth in the presence of IPTG on the induction of ADHII in recombinant organisms. (A) Recombinant *E. coli* S17-1(λ*pir*); (B) recombinant *Z. mobilis* CP4. Symbols: ○, pLOI706 EH/*adhB*; ●, pLOI706EH.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein compositions of whole cells, harvested at an OD_{550} of 0.5, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (25). Gels were stained with Coomassie blue.

Acetaldehyde analysis. Acetaldehyde was measured by gas chromatography with a Tracor model 560 gas chromatograph and a Chromosorb W HP (80/100 mesh) column at 90°C.

Enzymes and chemicals. Restriction enzymes and DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The *Eco*RI-*Hin*dIII multiple-cloning site from pUC18 was obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, N.J.), and the *Not*I linker was from New England Biolabs (Beverly, Mass.). *Taq* DNA polymerase and pBluescript II SK^+ were obtained from Stratagene (La Jolla, Calif.).

RESULTS

Vector construction. Plasmids pMMB66EH and pMMB66 HE are broad-host-range expression vectors which contain

TABLE 2. Expression of the *Z. mobilis* ethanologenic genes *adhA*, *adhB*, and *pdc* in *E. coli* S17-1(λ*pir*) and *Z. mobilis* CP4

Strain and construct	Orientation of gene	Concn of IPTG (mM)	Enzyme		Fold increase
			Type	Activity[a] (IU/mg of protein)	
S17-1(pLOI706EH/*adhB*)	Sense	0	ADHII[b]	0.46	1
S17-1(pLOI706EH/*adhB*)	Sense	2	ADHII[b]	30.4	66
CP4(pLOI706EH)[c]		5[d]	ADH	2.7	1
CP4(pLOI706EH/*adhB*)	Sense	0	ADH	7.4	2.7
CP4(pLOI706EH/*adhB*)	Sense	5	ADH	45.0	16.7
CP4(pLOI706HE/*adhB*)	Antisense	0	ADH	3.4	1.3
CP4(pLOI706HE/*adhB*)	Antisense	5	ADH	2.4	0.9
S17-1(pLOI706EH/*adhA*)	Sense	0	ADHI	0.25	1
S17-1(pLOI706EH/*adhA*)	Sense	2	ADHI	14.0	56
CP4(pLOI706EH)[c]		5[d]	ADHI	1.0	1
CP4(pLOI706EH/*adhA*)	Sense	0	ADHI	6.5	6.5
CP4(pLOI706EH/*adhA*)	Sense	5	ADHI	35.0	35.0
S17-1(pLOI706EH/*pdc*)	Sense	0	PDC	0.3	1
S17-1(pLOI706EH/*pdc*)	Sense	2	PDC	13.3	44.3
CP4(pLOI706EH)[c]		5[d]	PDC	4.9	1
CP4(pLOI706EH/*pdc*)	Sense	0	PDC	13	2.7
CP4(pLOI706EH/*pdc*)	Sense	5	PDC	31	6.3
CP4(pLOI706HE/*pdc*)	Antisense	0	PDC	4.6	0.94
CP4(pLOI706HE/*pdc*)	Antisense	5	PDC	5.3	1.1

[a] The listed activities are averages of three independent experiments. ADH activities were measured as a combination of ADHI and ADHII activity, with ADHII being the dominant enzyme. ADHI activity was measured in extracts containing 2-mercaptoethanol. Because of the rapid inactivation of ADHII in these extracts, the contribution of ADHII can be ignored.

[b] These recombinants lack the *adhA* gene.

[c] Recombinants containing plasmid pLOI706HE instead of pLOI706EH exhibited similar activities (less than 10% difference).

[d] Cells grown without IPTG exhibited similar activities (less than 10% difference).

an RSF1010 replicon, *tac* promoter, *lacI*^{q} repressor, and the *bla* gene as a selectable marker (18). The *Eco*RI-*Hin*dIII polylinkers in these vectors are present in opposite orientations. The polylinker is flanked at the 5′ end by the *tac* promoter and at the 3′ end by two strong transcriptional terminators of the *rrnB* gene. This polylinker region was modified to contain unique *Sst*I and *Not*I sites. The *Eco*RI-*Hin*dIII polylinker present in pMMB66EH and pMMB66HE was replaced by the *Eco*RI-*Hin*dIII polylinker from pUC18 to form pLOI704EH and pLOI704HE, respectively, resulting in the introduction of unique *Sst*I and *Sma*I sites. Next, the 12-bp *Sst*I-*Sma*I fragment of pLOI704EH was replaced by the 40-bp *Not*I-containing *Sst*I-*Sma*I polylinker fragment derived from pBluescript II SK$^+$ to form pLOI705EH. A *Not*I site was constructed in plasmid pLOI704HE by blunt-ended ligation of an 8-bp *Not*I linker into the *Sma*I site to produce pLOI705HE. Since *Z. mobilis* exhibits natural resistance to ampicillin (29), a *tet* gene was added to both constructs to facilitate selection. A *tet* gene was isolated on the 1,427-bp *Eco*RI-*Ava*I fragment from pBR322, treated with Klenow fragment, and inserted into the Klenow fragment-treated *Pvu*I site to produce pLOI706EH and pLOI706HE, respectively. This blunt-ended ligation destroyed the *Pvu*I site but restored the *Eco*RI site at the 5′ end of the *tet* gene. In pLOI706HE, transcription of the *tet* gene occurs in the same direction as the *tac* promoter but is inverted in pLOI706EH.

RSF1010-based vectors are typically present in low copy numbers (16). To facilitate the production of large amounts of plasmid DNA, our vectors were fused at the *Sst*I and *Not*I sites to plasmid pLOI142, a derivative of pUC18. (Plasmid pLOI142 was constructed by insertion of an 8-bp *Not*I linker into the *Sma*I site of pUC18.) The resulting plasmids, pLOI707EH (12,975 bp) and pLOI707HE (12,955 bp), contain the pUC18 ColE1 replicon in addition to the RSF1010 replicon and are present at a high copy number in *E. coli* (Fig. 1). These constructs, which now contain a functional *bla* gene (pUC18) in addition to the *tet* gene (Tc^r Ap^r), are double-duty vectors since they can be used for insertion of a foreign gene into the RSF1010-based controllable expression vector as well as into a modified pUC18 (pLOI142). By using Tc^r for selection, a series of RSF1010-based plasmids was constructed containing the ethanologenic genes pLOI706EH/*adhA*, pLOI706EH/*adhB*, pLOI706EH/*pdc*, pLOI706HE/*adhB*, and pLOI706HE/*pdc*. Ethanologenic genes in derivatives of plasmid pLOI706EH are transcribed in the same direction as the *tac* promoter, whereas derivatives of pLOI706HE contain these genes in reverse (antisense) orientation.

Regulated expression of *Z. mobilis* genes in *E. coli*. Expression of the *Z. mobilis adhA*, *adhB*, and *pdc* genes under control of the *tac* promoter was first examined in recombinants of *E. coli* S17-1(λ*pir*) (Fig. 2A; Table 2). ADHII was induced 66-fold by the addition of 2 mM IPTG. Similarly, ADHI was induced 56-fold and PDC was induced 44-fold. Maximal expression of all three genes occurred at IPTG concentrations of 1 mM and higher, although significant induction was observed in the range of 10 to 100 μM IPTG.

Regulated expression of plasmid-borne genes in *Z. mobilis*. In the absence of IPTG, *Z. mobilis* CP4 harboring plasmid pLOI706EH/*adhB* or pLOI706EH/*pdc* contained 2.7-fold-higher ADH and PDC activities than cells harboring unmodified pLOI706EH, whereas CP4(pLOI706EH/*adhA*) showed a 6.5-fold-higher ADHI activity (Table 2; Fig. 2B). Thus, *lacI*^{q} provided incomplete repression of the *tac* promoter in *Z. mobilis*. However, IPTG induction resulted in strong increases in expression: 35-fold for ADHI, 16.7-fold for ADHII, and 6.3-fold for PDC. Specific activities for pure preparations of these enzymes have been reported to be 240 IU of ADHI per mg (30), 950 IU of ADHII per mg (30), and

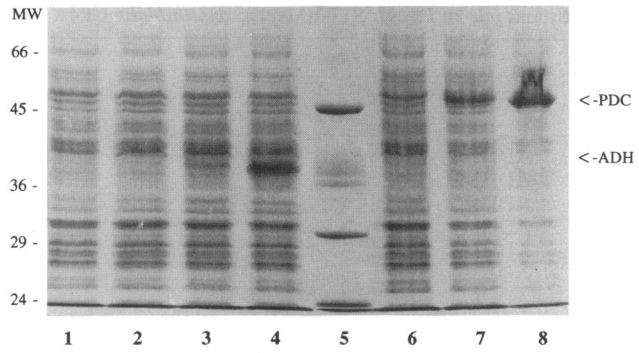

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis comparison of proteins in *Z. mobilis* CP4 recombinants grown in the presence and absence of IPTG (5 mM). Protein samples (10 μg of protein per lane) were loaded on a sodium dodecyl sulfate (2%) gel containing 12.5% acrylamide. Regions containing ADHII and PDC are marked by arrows. Molecular masses (in kilodaltons) are indicated to the left of the gel and consisted of bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa). Lanes: 1, CP4(pLOI706EH) + IPTG; 2, CP4(pLOI706HE/*adhB*) + IPTG; 3, CP4(pLOI706EH/*adhB*); 4, CP4(pLOI706EH/*adhB*) + IPTG; 5, reference proteins; 6, CP4(pLOI706HE/*pdc*) + IPTG; 7, CP4(pLOI706EH/*pdc*); 8, CP4(pLOI706EH/*pdc*) + IPTG.

85 to 181 IU of PDC per mg (6, 21, 31). On the basis of these values, the maximal amounts of ADHI, ADHII, and PDC proteins were estimated to be 14.5, 5, and 17% to 36% of total cellular protein, respectively. Expression of *adhA*, *adhB*, and *pdc* was fully induced at IPTG concentrations between 2 and 5 mM (Fig. 2B).

The specific activities of ADH and PDC in extracts of *Z. mobilis* CP4 recombinants containing the antisense constructs pLOI706HE/*adhB* or pLOI706HE/*pdc*, respectively, were similar to those observed in cells containing unmodified vector (Table 2). Induction of antisense RNA synthesis by IPTG also did not cause a significant reduction of the respective activities. This failure of antisense RNA to reduce translation in *Z. mobilis* may be related to the high abundance of *pdc* and *adhB* messages since both are highly expressed (28). The concentration of antisense RNA produced from pLOI706HE may be insufficient to suppress synthesis of ADHII or PDC.

This hypothesis was tested in part by studying the effect of overexpression of antisense *adhB* (pLOI706HE/*adhB*) on ADH activity in *E. coli* KO3 in which the *Z. mobilis pdc* and *adhB* genes are chromosomally integrated (32). The parental strain, *E. coli* B, contained a very low level of ADH activity (<0.004 IU/mg of protein). Strain KO3 exhibited an ADH activity of 0.15 IU/mg of protein, approximately 20-fold lower than that of *Z. mobilis* CP4 (Table 2). Introduction of plasmid pLOI706HE/*adhB* and uninduced expression of antisense *adhB* resulted in a reduction of ADH activity by 40% (0.09 IU/mg of protein). IPTG-induced expression of antisense *adhB* caused a further reduction of ADH activity (0.04 IU/mg of protein) to 27% of that observed without plasmid.

Proteins synthesized by *Z. mobilis* CP4 harboring pLOI 706EH/*adhA*, pLOI706HE/*adhB*, pLOI706EH/*pdc*, or pLOI 706HE/*pdc* were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). The ADHII protein was clearly evident as an overproduced protein in extracts of CP4(pLOI706EH/*adhB*). The observed increase in ADHII protein (Fig. 3, lanes 3 and 4) is consistent with the 6.1-fold increase in ADH activity measured after induction with IPTG (Table 2). PDC represents a prominent band in extracts of CP4(pLOI706EH) (Fig. 3, lane 1). This protein was elevated in CP4(pLOI706EH/*pdc*) without induction (lane 7) and increased further after growth in the presence of 5 mM IPTG (lane 8). The amount of PDC protein present after growth with IPTG appeared to exceed that estimated on the basis of specific activity (Table 2).

Effect of overexpression of fermentative genes on growth. The presence of the unmodified vector (pLOI706EH), uninduced pLOI706EH/*adhA*, or uninduced pLOI706/*adhB* caused a 10% reduction in the growth rate of *Z. mobilis* CP4 in comparison with cells lacking vector (Table 3). Induced expression of the plasmid-borne *adhA* or *adhB* gene caused a small further decrease in growth rate. In contrast, uninduced expression of *pdc* in CP4(pLOI706EH/*pdc*) resulted in a 22% decrease in growth rate. The addition of IPTG to this strain at the time of inoculation of seed cultures or immedi-

TABLE 3. Comparison of growth rates and enzyme and metabolic activities of *Z. mobilis* CP4 strains overproducing ADHI, ADHII, or PDC[a]

Strain and construct	Concn of IPTG[b] (mM)	Specific growth rate (h^{-1})	Q_{CO_2}[c] (IU/mg of protein)	Enzyme activity[d] (IU/mg of protein)		
				PDC	ADH[e]	G6PDH[f]
CP4	0[g]	0.51 ± 0.03	1.76 ± 0.06	6.5	3.0	2.5
CP4(pLOI706EH)	0[g]	0.46 ± 0.03	1.72 ± 0.06	4.9	3.0	2.2
CP4(pLOI706EH/*adhA*)	0	0.46 ± 0.03	1.71 ± 0.06	ND[h]	6.5	2.2
CP4(pLOI706EH/*adhA*)	5	0.42 ± 0.03	1.43 ± 0.06	ND	35.0	2.2
CP4(pLOI706EH/*adhB*)	0	0.46 ± 0.03	1.76 ± 0.06	4.9	8.8	2.1
CP4(pLOI706EH/*adhB*)	5	0.43 ± 0.03	1.56 ± 0.06	4.1	44.3	1.8
CP4(pLOI706EH/*pdc*)	0	0.40 ± 0.03	1.48 ± 0.06	13.6	2.7	2.2
CP4(pLOI706EH/*pdc*)[i]	5	0.30–0.09	0.60 ± 0.06	27.9	1.8	0.8

[a] All data are average values of two independent measurements.
[b] Induced cultures containing *adh* genes were precultivated with 5 mM IPTG.
[c] Values are averages of flux measurements at culture densities (OD_{550}) between 1.5 and 4.
[d] All cultures for enzyme assays were harvested at an OD_{550} of about 2.0 except for induced CP4(pLOI706EH/*pdc*), which was harvested at an OD_{550} of 0.56.
[e] See Materials and Methods for comments on ADH assays.
[f] Note that flux through this glycolytic enzyme is only half that measured as carbon dioxide evolution.
[g] Cells grown in the presence of 5 mM IPTG exhibited similar growth rates and enzyme activities (less than 10% difference).
[h] ND, not determined.
[i] Because of severe growth inhibition, CP4(pLOI706EH/*pdc*) was pregrown without IPTG but was induced at the time of dilution into fresh medium. Upon addition of IPTG, the specific growth rate of this culture decreased dramatically, ranging between the two values shown.

ately after dilution of overnight cultures into fresh medium caused a progressive decrease in growth rate to one-fifth that of wild-type CP4 (Table 3).

Effect of overexpression of fermentative genes on glycolytic flux in *Z. mobilis*. The metabolic activity of *Z. mobilis* CP4 strains was measured as Q_{CO_2} in complex medium containing 10% (wt/vol) glucose. After 100-fold dilution of a stationary-phase culture, Q_{CO_2} increased until the culture reached an OD of 0.8 to 1.3 while the growth rate remained constant (Table 3; Fig. 4). Metabolic activity remained near maximal between culture densities of 1.3 and 7 but rapidly declined at the end of the exponential growth phase. The highest Q_{CO_2} values ranged between 1.7 and 1.8 IU/mg of cell protein and were observed with *Z. mobilis* CP4, CP4(pLOI706EH), and the uninduced strains, CP4(pLOI706EH/*adhA*) and CP4(pLOI706EH/*adhB*). The Q_{CO_2} for uninduced CP4 (pLOI706EH/*pdc*) was 15% lower than that of the other recombinant strains.

In the first series of induction experiments with *adhA* and *adhB*, inocula for induced cultures were grown continuously in the presence of IPTG (Table 3). Overexpression of these genes caused small reductions in metabolic activity (16 and 11%, respectively). Strain CP4(pLOI706EH/*pdc*) was not grown continuously in the presence of IPTG because of severe growth inhibition but was induced at the time of dilution into fresh medium. Flux was less than half that of the parent strain and remained constant for at least 10 h, whereas the growth rate declined continuously.

In a second series of induction experiments, initial changes in enzyme activity, metabolic activity, and growth rate were analyzed during the period immediately after induction. For these experiments, IPTG was added to uninduced cultures functioning at maximal metabolic activity (Fig. 4A to C). As expected, ADHI, ADHII, and PDC activities increased rapidly in respective recombinants, with maximal expression after 4 h. Overexpression of *adhA* or *adhB* resulted in a small decrease in Q_{CO_2}. In contrast, overexpression of *pdc* caused more than 50% decline in flux, which mirrored the increase in PDC specific activity. With induced CP4(pLOI706EH/*pdc*), the onset of this decline occurred immediately in response to small increases in PDC activity. Surprisingly, growth rate was unaffected by the increases in ADHI, ADHII, or PDC (despite the large decline in flux associated with *pdc* induction).

Effect of overexpression of fermentative genes on other catabolic enzymes. The effect of overexpression of *adhA*, *adhB*, or *pdc* on the specific activities of other enzymes was measured (Table 3). Vector alone (pLOI706EH) resulted in 25 and 12% decreases in PDC and G6PDH activities, respectively, whereas ADH activity was unaffected. Full induction of *adhA* had no effect on G6PDH activity. Full induction of *adhB* resulted in 18 and 16% reductions of G6PDH and PDC activities, respectively, relative to those activities with unmodified vector.

In uninduced CP4(pLOI706EH/*pdc*), ADH activity was reduced by 10% but G6PDH activity remained unchanged. Full induction of *pdc* resulted in 40 and 64% reductions of ADH and G6PDH activities, respectively. These decreases in specific activity after full induction are consistent with the pronounced decline in the proportion of other cytoplasmic proteins in denaturing gels (Fig. 3, lane 8).

Acetaldehyde, a potentially inhibitory intermediate in ethanol fermentations, was measured in the broth of induced and uninduced cultures of CP4(pLOI706EH/*pdc*). The concentration of this compound remained between 1 and 2 mM independent of growth phase or IPTG induction, despite changes in PDC and ADH activities.

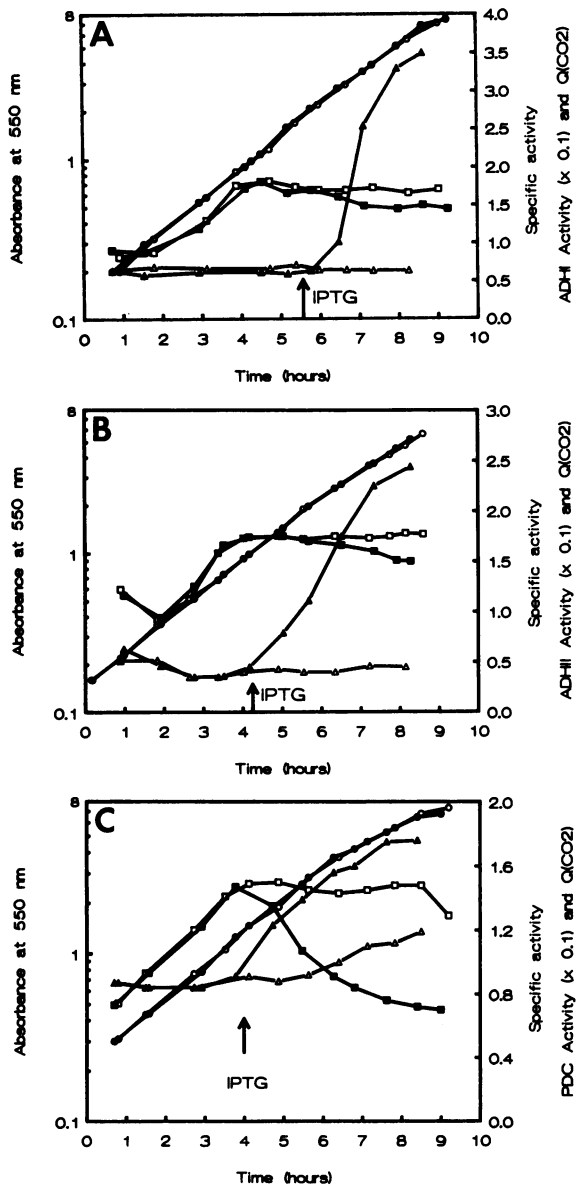

FIG. 4. Changes in growth, metabolic activity (Q_{CO_2}), and specific activities of ethanologenic enzymes during batch fermentation of *Z. mobilis* CP4 recombinants. (A) pLOI706EH/*adhA*; (B) pLOI706EH/*adhB*; (C) pLOI706EH/*pdc*. Duplicate cultures were grown in complex medium. Upon reaching maximal Q_{CO_2}, IPTG (5 mM) was added to one culture while the other served as an uninduced control. Open symbols, control culture without IPTG; filled symbols, cultures which were induced at the time point indicated (arrow). Enzyme activities and Q_{CO_2} are expressed in international units per milligram of protein. Symbols: ○, ●, growth; □, ■, Q_{CO_2}; △, ▲, ADH or PDC activity.

DISCUSSION

Regulated gene expression in *Z. mobilis*. To develop *Z. mobilis* as a model system for the study of glycolytic flux, a recombinant system is needed which will allow controlled gene expression. The *lacI*^{q} repressor and *tac* promoter

appear useful, although not ideal, for this purpose. In *Z. mobilis*, control was partial and expression was not completely blocked by the absence of inducer. The IPTG-independent expression in *Z. mobilis* may be related to codon usage since *lacI*^{q} contains five codons (GTG, GCG, TGT, CGA, CAA) which are rarely used for the synthesis of abundant *Z. mobilis* proteins (13, 24). The synthesis of *lacI*^{q} in *Z. mobilis* may be insufficient to saturate the *tac* operator sites.

Potentially, antisense RNA from plasmids could be used to down-regulate the expression of *Z. mobilis* chromosomal genes (43). Although this was not successful for *adhB* or *pdc* genes by using derivatives of pLOI706HE (Table 2), this approach did function to regulate chromosomal *Z. mobilis adhB* in *E. coli* KO3, a strain which harbors an integrated copy of the *Z. mobilis pdc* and *adhB* (32).

Effects of fermentative enzymes on glycolytic flux. Glycolytic flux measured as Q_{CO_2} increased during the growth of a diluted overnight culture, reaching a maximum value of 1.76 IU/mg of cell protein for wild-type CP4 (Fig. 4; Table 3). This increase in flux during growth agrees with previous results by Osman et al. (33), which also showed that the specific activities of most glycolytic and fermentative enzymes increase during the growth phase. In both investigations, the onset of decline in flux began at the end of the growth phase.

Kacser and Burns (22) and Heinrich and Rapoport (20) introduced the flux control coefficient to express in quantitive terms the extent to which an enzyme (E) in a metabolic pathway controls the flux (J) through that pathway. The flux control coefficient (C_E^J) is defined as the fractional change in pathway flux (dJ/J) divided by the fractional change in enzyme concentration or activity (dE/E) at a given set of J and E, usually the wild-type specific activity at maximal flux. In cells exhibiting maximal metabolic activity, our studies demonstrate that the specific activities of PDC, ADHI, and ADHII do not impede glycolytic flux. Relatively small changes in PDC activity (6.5 versus 4.9 IU/mg of protein) or in ADHI or ADHII activity (two- to threefold above the control specific activity) caused no significant increases in flux (Table 3). Thus, the flux control coefficients for ADHI, ADHII, and PDC at maximal metabolic activity can be regarded as near zero. Consequently, flux control in *Z. mobilis* under these conditions must be attributed to glucose uptake (15), other enzymes in glycolysis, or other metabolic processes. Similar studies with *Saccharomyces cerevisiae* have also shown that overexpression of *pdc* or *adh1* (at three- to fivefold) did not increase glycolytic flux (36) and that flux control in this organism is relegated to other aspects of metabolism (19).

Glycolytic flux in *Z. mobilis* CP4 recombinants decreased when the specific activity of individual fermentative enzymes was substantially increased above that present in control cells (Fig. 5). The onset of flux decline occurred at specific activities of around 6 to 8 IU/mg of cell protein for all three enzymes and was most pronounced with PDC. The flux control coefficient at these activities was estimated as the slope of a double-logarithmic plot of Q_{CO_2} versus the corresponding enzyme specific activity (Fig. 5). At the point of inflection for J and E, the C^J_{ADHI}, C^J_{ADHII}, and C^J_{PDC} values were estimated to be −0.2, −0.1, and −0.4, respectively.

The decline in flux was most dramatic for PDC. The onset of this decline extrapolated to very small changes in PDC activity, consistent with an initial enzymatic rather than bulk protein effect. At later times, large changes in PDC were

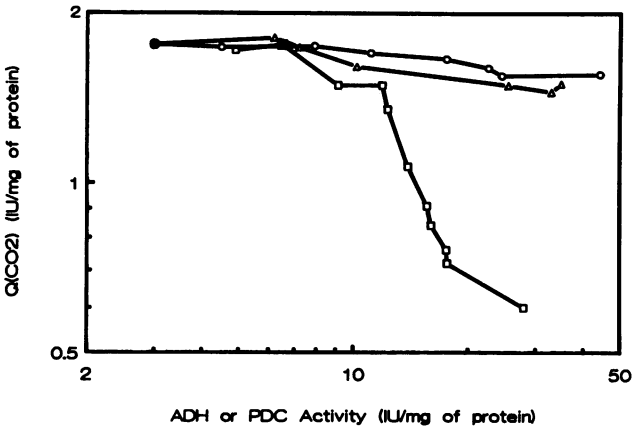

FIG. 5. Correlation between metabolic activity (Q_{CO_2}) and ADH or PDC activity in strains of *Z. mobilis* CP4. Double-logarithmic plots of Q_{CO_2} versus enzyme activity are shown. Flux control coefficients were calculated as the slope of each plot determined at the point of negative inflection. Symbols: △, ADH activity in recombinants containing plasmid pLOI706EH/*adhA*; ○, ADH activity in recombinants containing pLOI706HE/*adhB*; □, PDC activity in recombinants containing pLOI706EH/*pdc*. Solid symbols represent Q_{CO_2} at wild-type activities of ADH (ADHI plus ADHII) and PDC in *Z. mobilis* CP4.

accompanied by a reduction in the specific activities of other glycolytic enzymes which could contribute to a further decline in flux. The concentration of acetaldehyde, which is known to inhibit growth at concentrations above 10 mM (42), was low and did not change during induction of PDC, despite the concomitant decrease in ADH activity. Maintenance of a near-constant level of acetaldehyde during changes in PDC and ADH activities implies a tight coupling between synthesis and utilization, perhaps by the intracellular concentrations of NAD^+ and NADH.

Effects of fermentative enzyme levels on growth. IPTG induction of PDC in cells in which metabolic activity was only half maximal (IPTG addition upon dilution of an overnight culture into fresh medium) resulted in an immediate decline in growth rate. This decline was not found to result from a decrease in glycolytic flux and ATP generation. Furthermore, it is unlikely that this decline results from a bulk effect of increased PDC protein, since the onset occurred immediately, prior to protein accumulation. Under these conditions, the immediate decline in growth may result from the starvation of biosynthesis for carbon skeletons due to increased conversion of pyruvate into acetaldehyde by excessive PDC.

Overexpression of all three ethanologenic genes examined caused a reduction in growth rate after several generations (Table 3). However, for cells functioning near the maximal metabolic rate, no reduction in growth rate was observed during the initial period after induction (Fig. 3 and 4) despite a 50% decrease in glycolytic flux in induced cultures of CP4(pLOI706EH/*pdc*). Conversely, the twofold increase in flux observed during growth after dilution of an overnight culture into fresh medium was not accompanied by any increase in growth rate (Fig. 4). Since the ED pathway is the least-efficient glycolytic pathway known (net yield of 1 ATP per glucose metabolized), near-maximal rates of glycolytic flux have generally been regarded as essential to support *Z. mobilis* growth at a doubling rate of less than 2 h (8, 33). However, our results indicate that the rate of glycolysis and

ATP production in cells functioning at maximal flux exceeds biosynthetic needs for growth and homeostasis by at least twofold in rich medium. Thus, in *Z. mobilis*, an imperfect coupling must exist between ATP generation and biosynthesis. Under conditions of maximal flux, some alternative route must be available to dissipate excess ATP (23). Although the nature of this energy dissipation system is unknown, flagellar motion in *Z. mobilis* represents an obvious candidate.

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